Extract from Document **C. H. 398** *published by the Health Organisation of the League of Nations:*

THE BIOLOGICAL STANDARDISATION OF INSULIN

8.

THE STANDARDISATION OF INSULIN BY THE DETERMINATION OF THE CONVULSIVE DOSE FOR MICE

By J. W. Trevan and Ellen Boock
(Wellcome Physiological Research Laboratories, Beckenham, Kent).

The obvious simplicity of using convulsions as an indicator in the assay of insulin has led to the development of several "convulsion methods"; one, of which the most complete description has been published, is that of Voegtlin. We have been using mice for the standardisation of insulin since the early part of 1923, and have gradually arrived at the method described below in detail in which some 30 mice of a given stock are injected with a standard sample of insulin and 30 others with the batch of insulin under investigation. The animals are kept during the test at 38°C. and the comparison of the percentages showing convulsions within a limited time enables a determination of the activity of the batch under test to be made. The method gives results parallel to those of the rabbit test and we use it as a routine for all our tests.

Method.

Mice are fed, preferably for at least a week after receipt, on white bread and excess of milk. They are kept in batches of 200 in large cages, 5′ × 3′ × 1′, with perforated zinc tops. The animals required are removed to an empty cage at 5 p.m. on the day previous to a test and deprived of food. During the test, which is made at any time from 9 a.m. to 4 p.m., the mice are kept in zinc boxes about 5″ cube, which are for three-quarters of their height immersed in a water bath at 38°C. The boxes are covered with glass lids and perforations are made around the top of the walls for ventilation (Fig. 1). The apparatus should be shielded from draughts. The mice are injected subcutaneously with a dilution of insulin in a dose of 0.0075 to 0.01 clinical units per 20-gram body-weight. Immediately after the injection the mice are transferred to the heated boxes, six mice in a box, of which three are injected with the insulin, the activity of which is under investigation, and three with a dose of some standard sample of insulin. Sixty mice are put up at each test and four tests in all are made. The number of animals which show symptoms or die during two hours is then

S. d. N. (réimp.) 7/26. Imp. Kundig.

observed by inspecting the boxes at ½, ¾, 1, 1½, and 2 hours. Most of the mice developing symptoms of hypoglycæmia do so in the first hour. The symptoms vary from violent convulsions down to muscular atony. There is some difficulty in determining the minor degrees of hypoglycæmic effect and in cases of doubt we lay the mouse on its back. If it does not turn over immediately

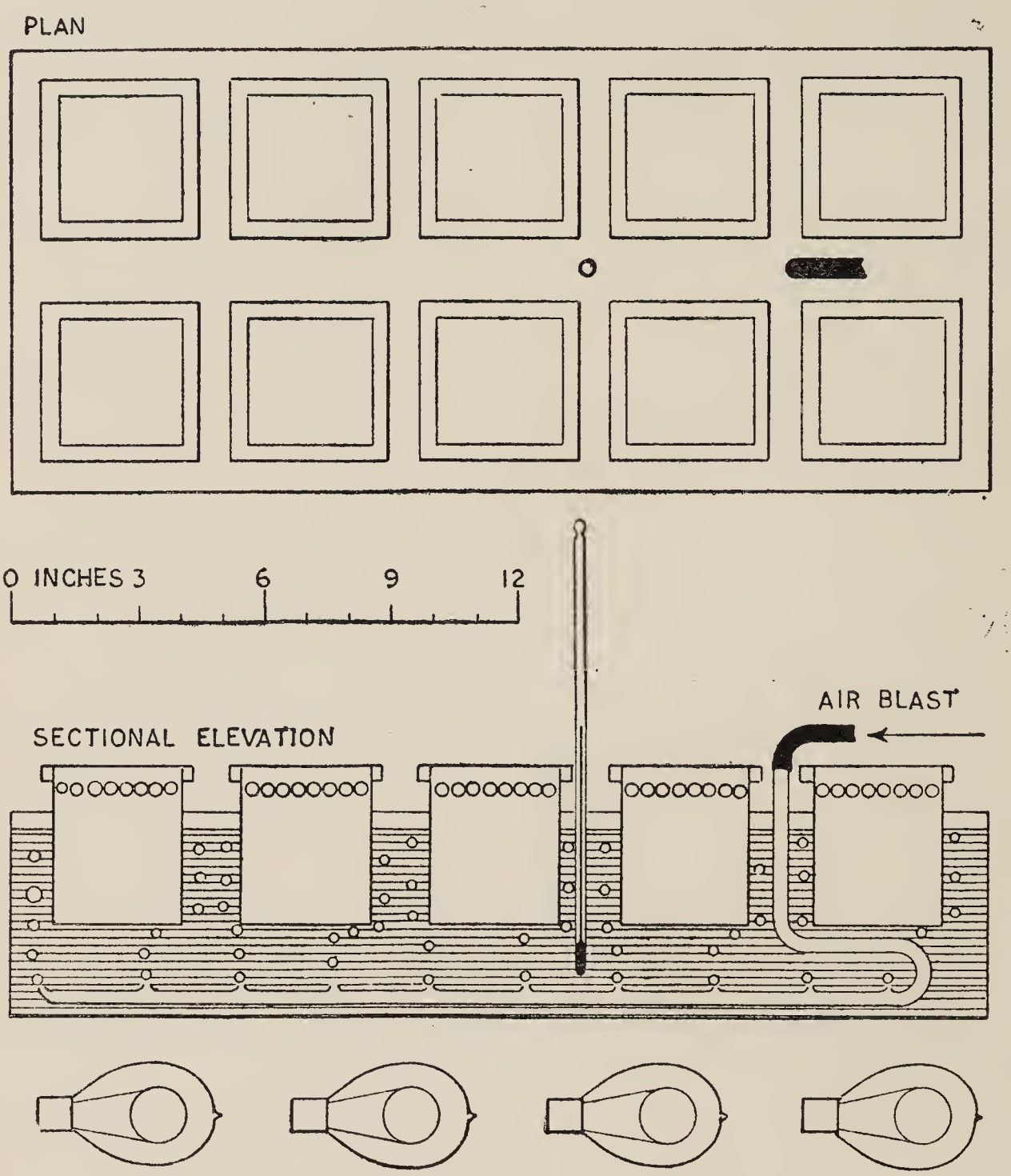

FIGURE 1.
Apparatus for keeping mice at 38°C.; described in text.

we consider it to show "symptoms". The intravenous injection of glucose causes the animal to recover immediately. Examination of the mice must be made rapidly, to prevent fall of temperature.

Table 1 gives the results of a series of experiments with the new International Standard, referred to as "New Standard", and with the dry preparation in current use in England as a standard, referred to as "Old Standard". The first column gives the dose of the powder in thousandths of a milligram, used for a 20-gram

mouse, the size of the dose administered being in direct proportion to the weight of the animal. Subcutaneous injection was used in all cases, suitable dilutions with saline being made so that 0.5 c.c. was injected for 20-gram body-weight.

In Experiments 1 and 4, simultaneous experiments with the new and the old standards were made. In Experiments 5 and 6, all the mice were injected with the new standard with the object in view of establishing on a large number of animals the form of the dose-convulsion curve. The experiments were each done on a different

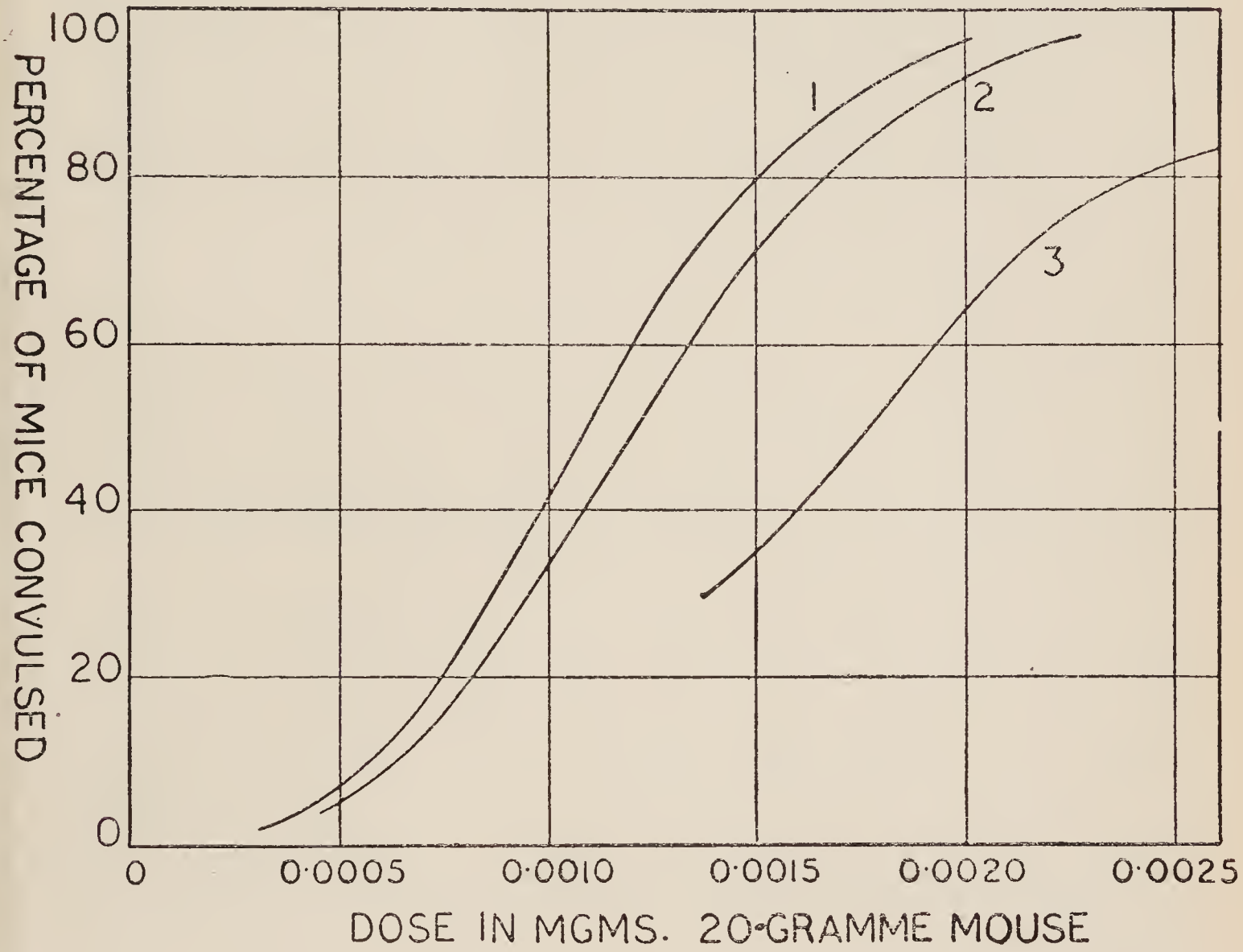

FIGURE 2.

Convulsion-dose curves at 38°C. for the international standard insulin (1), and the current English standard (2), from the figures of Experiment 4. The abscissæ of curve 2 are 10 per cent larger than those of curve 1. Curve 3 is the same as curve 1 except that the abscissæ are increased by 60 per cent. It fits the points obtained in Experiment 3, and illustrates the mass variation of the whole stock of mice which occurs at times. As will be seen from the table, although the susceptibility of the mice on this occasion was very much less than the average, the calculated ratio between the activities of the two samples of insulin was substantially the same as on the days when the susceptibility of the mice was much higher.

date, the investigation spreading over about six weeks. Under "mice convulsing", the numbers of mice convulsing are given in the form of fractions, the numerator representing the number showing symptoms and the denominator the total number injected.

The mice represented in each horizontal line in Experiments 1 to 4 were chosen haphazard from the same group and were arranged after injection so that each box contained three injected with the new and three with the old standard in order to ensure as far as possible equality of temperature and other conditions. Some of the results obtained are plotted in Fig. 2. These figures show that the percentage of mice which develop convulsions with a given dose varies on different days. For this reason, the mouse-convulsive dose can only be used for the comparison of the relative activities of two samples of insulin, and figures for the relative activity are calculated from the respective convulsion rates for the two samples when injected at the same time by a method which depends on the following considerations. In Fig. 2, the full-line curves 1 to 3 are all the same curve drawn to different abscissal scales. It will be seen that each of the curves fits one or other of the series of points obtained with a fair degree of accuracy. Curve 1 fits the points obtained in Experiments 4 and 5 for the "new" standard; Curve 2 the points obtained for the "old" standard in Experiment 4; Curve 3 those for the "old" standard in Experiment 3. The actual deviations between the smoothed curve and the observed points are, of course, due to the statistical error of sampling, which will be discussed later, but the smoothed curves may be taken to represent with sufficient accuracy the points which would be obtained if indefinitely large groups of animals could have been used to establish them.

Since the difference between each curve is only one of abscissal scales, it follows that for all the experiments the relation between *percentage* increment of dose and percentage increment of convulsion rate is constant, when sufficiently large groups of mice are taken. For example, with Curve 3, raising the dose from 1.6 to 2.4 (a rise of 50 per cent) caused an increase in convulsion rate from 40 to 80 per cent. With Curve 1, to produce an increase in the convulsion rate from 40 to 80 per cent the necessary dose rises from 1.0 to 1.5, which is the same percentage increase in dose as before.

Similar results will be obtained with any other pair of convulsion rates. The interpretation we place upon this is the following: The curves each represent integrated frequency curves of the variation in susceptibility of the individual mice in any one group. The slope of the curve when drawn to a scale taking 50 per cent mortality as unit dose is a function of the coefficient of variation of the susceptibility of individual animals. The figures show that day-to-day variations affect the mean individual susceptibility but do not affect appreciably the distribution of the individual susceptibilities around the mean, *i.e.*, the coefficient of variation is unchanged. The variation of the factors, whatever they are, which cause periodic variation in the mean susceptibility is completely uncorrelated with the individual susceptibility of the animal.

As a result of these and other similar observations, we take it as established that the shape of the convulsion-dose curve is constant

for all groups of mice treated as described, although the absolute values of the abscissæ vary, and we adopt the following procedure for the calculation of the relative activity of two solutions of insulin which have given different convulsion rates on two groups of mice injected at the same time. A curve such as 1, obtained by the simultaneous injection of a large group of mice on the same day, is constructed and used as a reference curve. Then, although this curve does not give for every group of mice the absolute values of the activity of a dose of insulin which causes any observed proportion of convulsions, it can be used to calculate from the convulsion rates obtained the ratio of the activities of doses of any two samples of insulin injected into two groups of mice at the same time. In Experiment 1 *a*, as an example, the ratio of activities of the two is estimated by taking the reference curve (such as Curve 1) and reading off from the curve the doses which, on the date on which the curve was made, give mortalities of 6 and 8 respectively out of 30. The doses giving this convulsion rate in the group from which Curve 1 was made were 0.75×10^{-6} grams and 0.805×10^{-6}, a ratio of 1.00 to 1.07. Using any one of the other curves, the proportion will, of course, be the same since the different curves differ only in abscissal scale. Since the "new" and the "old" standards were injected in doses of the same weight in the experiment under discussion (1*a*), the activity of the "new" standard is 1.07 times that of the "old" according to this particular experiment. In Experiment 2*a*, with a larger dose of each, 15 out of 30 convulsed with the "old" and 18 with the "new" standard. Fifty per cent mortality when Curve 1 was obtained corresponded to 1.1×10^{-6} and 60 per cent to 1.2×10^{-6}, a ratio of 1 to 1.09. The activity of the "new" standard therefore is 1.09 times that of the "old' standard according to Experiment 2*a*. In such a manner were all the figures in the last column obtained. The discrepancies amongst them are, by pure chance, smaller than are to be expected in such a series. That there are discrepancies chiefly depends on the inevitable error of sampling. If 50 per cent of a very large group of animals would be thrown into convulsions by a given dose "*a*" per unit weight of mouse, the probability is small that "*a*" would cause 15 to convulse out of a group of 30 chosen haphazard from the large group. The probability that the deviations from the true value due to the small size of the sample will be not greater than any given value can be readily worked out from the standard probability formula. The discrepancy produced in the final evaluation of the ratio between two specimens of insulin in the manner laid down above depends not only on the deviations in convulsion rate due to sampling errors but also on the slope of the convulsion-dose curve. If the convulsion-dose curve were nearly perpendicular to the abscissa, any deviation due to sampling would alter the final value assigned to the ratio very little. With a sloping curve such as the one actually obtained for insulin, the error is considerable. It is approximately 360 to 1 (corresponding to three times the standard deviation) that the error of the comparative values assigned when

using this test for insulin will not exceed 34 per cent with a 75 per cent convulsion rate when 30 mice are used in each group. This figure is obtained by determining from the curve the increment of dose which corresponds to the standard error involved in using two groups of 30 mice each. The whole question of the accuracy of determination of toxicity is being made the subject of a separate paper. The majority of the estimations will, of course, fall between closer limits than these, as is the case in the series of experiments under consideration. The average value of the "new" standard as a percentage of the "old" as given by these tests is 110 per cent. Since 490 animals were injected in each group altogether, the non-significant range of error is of the order of 10 per cent (calculated graphically). The difference obtained is therefore of the order of difference which is not significant, but the result indicates that the activity of the "new" standard is almost certainly not less than 100 per cent nor greater than 120 per cert of the "old" standard. The standard error for the figures in the last column of Table 1 was also worked out by the root mean-square method, and gives 6.2 per cent as the significant range, but the higher figure given above is probably more accurate.

The apparent difference in the two samples is shown graphically in Fig. 2. The points obtained for the "new" and the "old" standard in Experiment 4 lie along Curves 1 and 2 respectively, of which Curve 2 is constructed with abscissæ 10 per cent greater than Curve 1.

We have investigated various factors which might interfere with the use of the method.

Temperature at which experiment is carried out. It was due to private information received through Dr. Dale from Prof. Krogh that we tried keeping the animals warm after injection. He recommended 28°C., but we have found that better results are obtained by keeping the boxes at 38°C. (Fig. 3). The shape of the convulsion-dose curve as given (for 28°C.) is only approximate because of the statistical difficulties of obtaining points for such a flat curve, but it represents the order of difference from the 38° curve. The difference in shape indicates that the less sensitive animals are more affected by the low surrounding temperature than the more sensitive. It is obviously quite hopeless to attempt to standardise insulin on our mice at room temperature, or even at 28°C, and comparisons by the method described above are invalidated if the temperature at which the comparison is made differs from the temperature at which the standard curve was made.

The explanation we offer of the effect of temperature is the following: The temperature of mice is variable (see Table 2), and unless the body temperature is kept at 37° convulsions do not develop when the sugar falls. In addition, the fall in the blood-sugar is often accompanied by a fall in body temperature (see Table 2), which aggravates the effect. By keeping the animal at 38°C. the body temperature is maintained. Huxley has shown that the rate of action of insulin in cold-blooded animals increases with the temperature. The temperature during the first half-hour

or so is the most important. We injected 50 mice at 29°C. with insulin and 50 at 38°C. In the first group one convulsed in one hour, in the second group 32. When the temperature of the first group was raised to 38°C. in the second hour, 23 mice altogether convulsed. We have the impression that it is better not

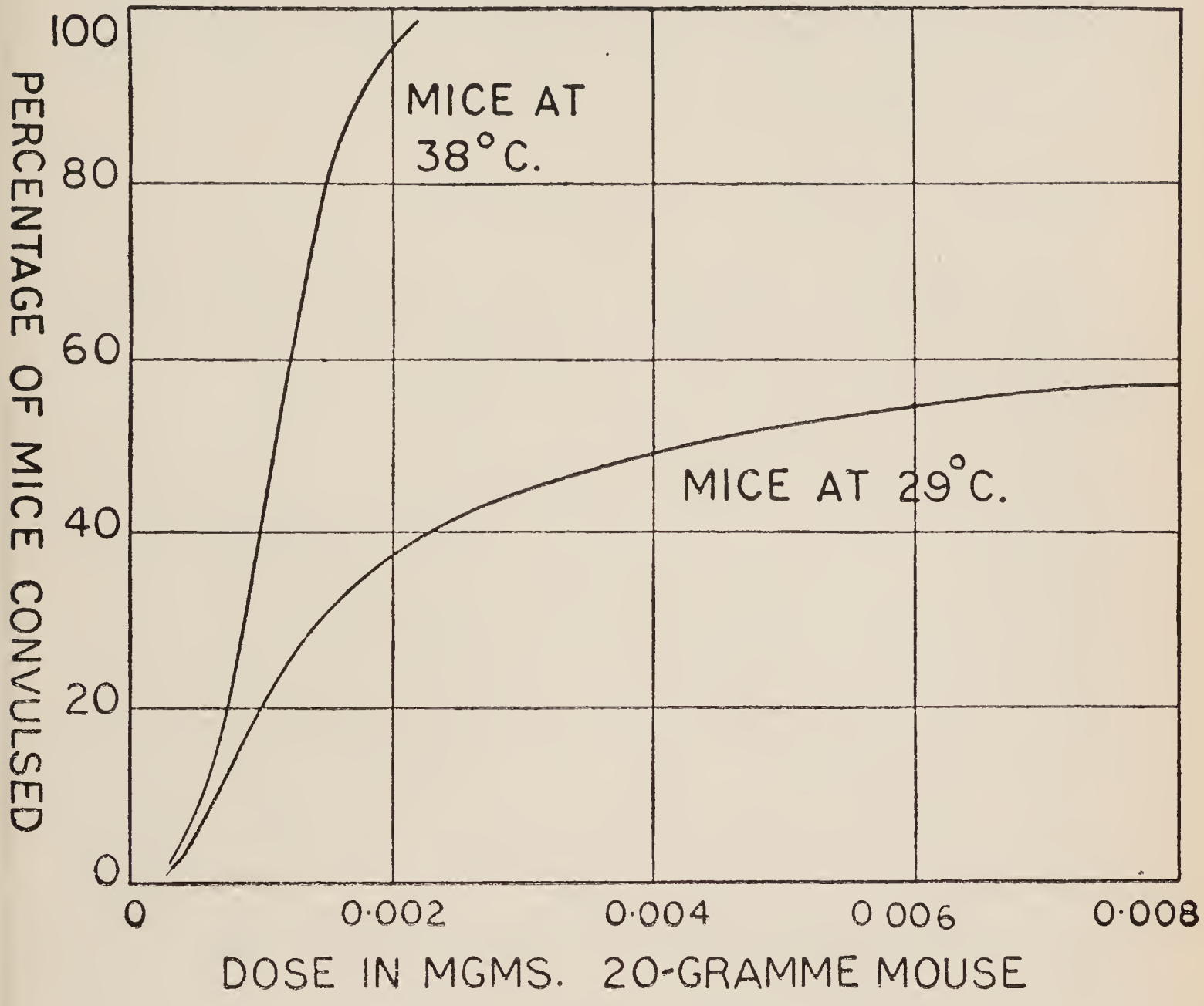

FIGURE 3.

Convulsion-dose curves for mice injected with insulin at room temperatures of 38°C. and 29°C. respectively.

to warm the mice before injecting them, as the mice which have been long in the boxes at 38°C. are rather less sensitive than those which have not been previously heated. The temperature of such a small object as the mouse rises very rapidly to that of its surroundings at 38°C.

Diet. Bainbridge has shown in these laboratories that increase of the ratio between fat and carbohydrate in the diet diminishes the sensitiveness of mice and rats to insulin. We have found that for three or four hours after the last meal the sensitiveness of mice is decreased, but after this, and up to 22 hours at least, no evidence of alteration of sensitiveness can be obtained (Table 3).

Weight. Table 5 gives the result of an analysis of figures obtained over some months. Animals weighing less than 15 grams are slightly more sensitive than those weighing more. The difference has been ignored.

Colour. The colour of mice has no significant effect on sensitiveness (Table 4).

Route of injection. The choice of intravenous, subcutaneous or intraperitoneal injection has no effect in mice on the number convulsing, although convulsions occur sooner when the route chosen is the intravenous one.

Discussion.

One of the factors which led us to the adoption of this method was the necessity for dealing with large numbers of tests for experimental purposes. For preliminary tests we use smaller groups and for rough estimates 10 mice are sufficient. A single worker, with the assistance of one boy, can do rough tests of 15 samples in a day, using 10 mice per group, or five samples using 30 mice in each group. The method has also the advantage that less technical skill is required than for the performance of the rabbit test. On the other hand, as has been pointed out to us by Marks, the method has the theoretical disadvantage that the degree of hypoglycæmia is not estimated directly but only by the occurrence of convulsions, and it is well known that convulsions in the rabbit, and probably in the mouse at room temperature, are not an inevitable consequence of any given degree of hypoglycæmia. We have stated reasons for supposing that the effect of raising the temperature of mice to 38° is to increase the number of animals which convulse when the blood-sugar falls, but we recognise that the conditions at 38° are probably not such that convulsions *always* follow the same degree of hypoglycæmia. The lack of correlation between convulsions and hypoglycæmia would render the convulsion test much less useful than the direct measurement of blood-sugar, were it not that the number of independently variable factors which affect the degree to which the blood-sugar falls is already so large that the effect of the addition of one other variable, namely, the variability of the convulsive response to hypoglycæmia, can be compensated by a reasonable increase in the number of animals used. We have found good agreement within the limits of experimental error between the rabbit method and the method described above, and the possibility of obtaining comparable results by different methods on two species of animals adds to the confidence with which the results obtained by either method can be regarded.

Summary.

A method for the comparison of different samples of insulin using a convulsive dose for the mouse as an indicator is described. Using this method, the international standard is estimated to

contain 8.8 clinical units per milligram, with a standard deviation of 0.266, assuming the current standard to contain 8 clinical units per milligram.

BAINBRIDGE: *Journ. of Physiology* (in the press).
HUXLEY: *Nature*, 1923, 113.
VOEGTLIN, DUNN and THOMPSON: Public Health Reports, August 1924; 39, 1935.

TABLE 1.

Exp. No.		Dose per 20-gram mouse Grams × 10-6	Mice convulsed Old standard No.	Per cent	New standard No.	Per cent	New standard as percentage of old
1	*a*	0.94	6/30	20	8/30	26.6	107
	b	1.25	15/30	50	18/30	60	109
2	*a*	1.6	14/30	46.6	18/30	60	119
	b	1.6	14/30	46.6	15/30	50	107
3	*a*	1.6	12/30	40	12/30	40	100
	b	1.9	18/30	53.3	20/30	66.6	115
	c	2.2	19/30	63.3	22/30	73.3	108
4	*a*	0.94	20/60	33.3	24/60	40	113
	b	1.25	34/60	56.6	39/60	65	109
	c	1.6	20/30	66.6	25/30	83.3	126
	d	1.9	26/30	86.6	27/30	90	105.5
5	*a*	0.625			10/30	33.3	
	b	0.94			14/30	46.6	
	c	1.25			19/30	63.3	
	d	1.57			26/30	86.6	
	e	1.875			28/30	93.3	
	f	2.2			29/30	96.6	
6	*a*	0.78			4/30	13.3	
	b	0.94			10/30	33.3	
	c	1.1			14/30	46.6	
	d	1.25			36/60	60	
	e	1.4			40/60	69.6	
	f	1.6			49/60	81.6	
	g	1.7			54/60	90	
	h	1.875			28/30	93.3	

TABLE 2.

	External temperature	Body temperature of mice	Number of mice	Number of mice convulsed
Normal	19°	34.8°	20	
After Insulin . .	19°	31.2°	20	3
Normal	22°	32.8°	17	
After Insulin . .	22°	30.5°	17	0
Normal	38°	37.95°	30	
After Insulin . .	38°	37.1°	27*	15

* 3 dead.

Table 3.

Effect of Feeding on the Response of Mice to Insulin.

No food since previous day. Standard dose.	Food not removed. Standard dose.	Just fed. Standard dose.	Just fed. 1.14 standard dose.
21/30	16/30	4/30	10/30

Table 4.

White	Black	Black and white	Other colours	*Total*
24/33 = 73%	11/15 = 73.5%	33/45 = 73.5%	124/167 = 74.3%	192/260 = 73.9%

Table 5.

Effect of Weight on Response of Mice to Insulin.

Total convulsed	Under 15 grams	15 grams and over
184/251 = 73.3%	93/124 = 75%	91/127 = 71.5%